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In vivo Inhibition of Serine Protease Processing Requires a High Fractional Inhibition of Cathepsin C

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Running Title: Cathepsin C-dependent serine protease processing

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Abbreviations: AMC, aminomethyl coumarin; CG, cathepsin G; DMK, diazomethyl ketone; Dpa, N-3-(2-4-dinitrophenyl) 1-2-3-diaminopropyonyl; EOA, enzyme occupancy assay; FBS, fetal bovine serum; gB, granzyme B; GF-DMK, Gly-Phe-DMK; GM-CSF, granulocyte-macrophage colony stimulating factor; IL, interleukin; L2p, Lysyl-2-(picolinoyl); Mca, (7-methoxycoumarin-4-yl)acetyl; MES, 2-(N-Morpholino)ethanesulfonic acid; MPO, myeloperoxidase; NE, neutrophil elastase; PBS, phosphate buffered saline; PLS, Papillon-Lefèvre syndrome; Pr-3, proteinase-3; SEM standard error of the mean; TBS, Tris-buffered saline

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Abstract

Inhibition of cathepsin C, a dipeptidyl peptidase that activates many serine proteases, represents an attractive therapeutic strategy for inflammatory diseases with a high neutrophil burden. We recently showed the feasibility of blocking the activation of neutrophil elastase, cathepsin G and proteinase-3 with a single cathepsin C selective inhibitor, in cultured cells. Here we measured the fractional inhibition of cathepsin C that is required for blockade of downstream serine protease processing, in cell-based assays and *in vivo*. Using a radiolabeled active site probe and U937 cells, a 50% reduction of cathepsin G processing required ~50% of cathepsin C active sites to be occupied by an inhibitor. In EcoM-G cells, inhibition of 50% of neutrophil elastase activity required ~80% occupancy. Both of these serine proteases were fully inhibited at full cathepsin C active site occupancy, whereas granzyme B processing in TALL-104 cells was partially inhibited, despite complete occupancy. *In vivo*, leukocytes from cathepsin C +/- mice exhibited comparable levels of neutrophil elastase activity to wild-type animals, even though their cathepsin C activity was reduced by half. The chronic administration of a cathepsin C inhibitor to rats, at doses that resulted in the near complete blockade of cathepsin C active sites in bone marrow, caused significant reductions of neutrophil elastase, cathepsin G and proteinase-3 activities. Our results demonstrate that the inhibition of cathepsin C leads to a decrease of activity of multiple serine proteases involved in inflammation, but also suggest that high fractional inhibition is necessary to reach therapeutically significant effects.

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Introduction

Cathepsin C (E.C.3.4.14.1) is a cysteine protease with dipeptidyl aminopeptidase activity that activates several pro-inflammatory serine proteases by removal of an amino-terminal inhibitory dipeptide (McDonald et al., 1969; McGuire et al., 1992; McGuire et al., 1993; Pham and Ley, 1999; Wolters et al., 2001; Sheth et al., 2003; Adkison et al., 2004). Cathepsin C mutations in humans are associated with Papillon-Lefèvre syndrome (PLS, MIM#245000), a rare autosomal recessive disorder that is characterized by palmoplantar keratosis and severe pre-pubertal periodontal disease (Toomes et al., 1999; Hart et al., 1999). In these patients, reductions (>95%) of cathepsin C activity result in complete or near complete loss of the enzymatic activities of the neutral serine proteases cathepsin G (CG), neutrophil elastase (NE) and proteinase-3 (Pr-3; Pham et al., 2004; deHaar et al., 2004) in neutrophils, and granzyme B (gB) in resting NK cells (Meade et al., 2006). Approximately a fifth of PLS patients exhibit increased susceptibility to infections (Almuneef et al., 2003), although it is not clear whether this is solely due to lack of neutral serine protease activity. The reduction of serine protease activities was also observed in cathepsin $C^{-/-}$ mice, which showed considerably lowered levels of NE, CG and Pr-3 (Adkison et al., 2002). In addition, the enzymatic activities of mast cell chymases (mMCP1, 2, 4 5 and 9) and mast cell tryptase (mMCP6) were strongly reduced (Wolters et al., 2001). Granzymes A and B from cathepsin $C^{-/-}$ murine LAK cells were incorrectly processed at their N-termini and consequently, were inactive and unable to mediate target cell killing *in vitro* (Pham and Ley, 1999). The secondary deficiency of neutral serine proteases in cathepsin $C^{-/-}$ mice largely explains the improved resistance of these mice to collagen-antibody induced arthritis (Adkison et al., 2002). These

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observations validate cathepsin C as a interesting target for therapeutic intervention for diseases where serine protease contribute to the pathophysiology, but also impose necessary caution on the possible risks associated with complete cathepsin C inhibition.

Cathepsin C loss-of-function mutations have provided invaluable knowledge of its role in serine protease activation and in their role in inflammation. However, little information is available under circumstances where cathepsin C activity is partially reduced. We recently described potent reversible dipeptide nitrile inhibitors of cathepsin C that blocked the cathepsin C-mediated activation of Pr-3, CG and NE in cells (Méthot et al., 2007). Here, we investigated the effects of partial cathepsin C inhibition on serine protease activation in cultured cells and *in vivo*. We used these reversible cathepsin C inhibitors in combination with a radiolabeled active site probe to measure the percentage of cathepsin C blocked by the inhibitors and the functional consequence on serine protease activation. Our results suggest that fractional inhibition requirements for processing of downstream serine protease vary with cell type and target serine protease, but in general, a high degree of cathepsin C inhibition is required for a functional effect. Cathepsin C fractional inhibition requirements were also investigated *in vivo*. Peripheral blood leukocytes of cathepsin $C^{+/-}$ mice contained the wild type levels of CG and NE activities, despite a 50% reduction in cathepsin C enzyme activity. These results strongly suggest that greater than 50% of cathepsin C inhibition will be needed for therapeutic success. Indeed, we could show that partial inhibition of CG, Pr-3 and NE was achieved in peripheral blood leukocytes of rats chronically infused with a cathepsin C inhibitor,

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but at the requirement of a very high cathepsin C active site occupancy in the bone marrow.

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Materials and Methods

Reagents - Gly-Phe-DMK was purchased from ICN Pharmaceuticals, Aurora OH. Ala-4-(I)Phe-DMK, Ala-4-(^{125}I)Phe-DMK (Méthot et al., 2007) and dipeptide nitriles were synthesized at Merck Frosst. A detailed description of their synthesis will be published elsewhere (DG). All dipeptide nitrile cathepsin C inhibitors are trifluoroacetic acid salts. Details on the properties of cmpd 1 have been published in Méthot et al. (2007). Details on the caspase inhibitor M867 can be found in Méthot et al (2004). The gB inhibitor compound 20 (Willoughby et al., 2002) and the NE inhibitor L-694,458 (Davies et al., 1991) were obtained from Merck & Co. Aprotinin-agarose and β-estradiol were purchased from Sigma-Aldrich. MeOSuc-Ala-Ala-Pro-Val-AMC and Suc-Ala-Ala-Pro-Phe-pNA were obtained from Bachem AG, and NH_2-Gly-Arg-AMC from Nova Biochem. Mca-L2p-Tyr-Asp-Ala-Lys-Gly-Asp-$DpaNH_2$ was custom-synthesized at AnaSpec Inc., San Jose. Methionine, glutamine-free RPMI1640 and mouse recombinant GM-CSF were obtained from Biosource International. Recombinant human IL-2 and IL-12 were purchased from eBioscience. RPMI1640, IMDM, Na-pyruvate, penicillin-streptomycin and PBS solutions were from Mediatech Inc., FBS was from Hyclone. ^{35}S-methionine (1000 Ci/mmole) was obtained from Amersham Biosciences.

Serine-protease cell-based assays – The EcoM-G and U937 cell-based assays have been described in detail in Méthot et al., 2007. Briefly, CG processing in U937 cells was measured by pulse-chase and aprotinin-agarose binding. Exponentially growing U937 (ATCC CRL-1593.2) cells were seeded at 2 x 10^6 cells/ml in methionine and glutamine-

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free RPMI1640 supplemented with 10% dialyzed FBS, and starved for 30 min in the presence of the cathepsin C inhibitors before the addition of ^{35}S-methionine (10 μCi/ml). The cells were pulsed for 30 min, washed twice and re-seeded in complete U937 culture media (RPMI1640 containing 10% FBS, 100U/ml each of penicillin and streptomycin, 1 mM Na-pyruvate and 10 mM Hepes). Cathepsin C inhibitors were freshly added and the cells were incubated for an additional 3 h. After the chase period, the cells were washed with PBS and lysed in lysis buffer A (50 mM Tris-HCl pH 8.0; 100 mM NaCl; 1% NP40). Cell debris was removed by centrifugation at 15,000 x g for 10 min. The supernatant was mixed with 4 volumes of binding buffer A (50 mM Tris-HCl pH 8.0, 1 M NaCl, 1% NP40) and aprotinin-agarose beads (Sigma) and incubated for 1 h at room temperature with gentle rotation. The beads were pelleted by brief centrifugation at 750 x g then washed 3 times with binding buffer A and once with lysis buffer A. Aprotinin-agarose-bound proteins were resolved on 10-20% SDS-PAGE gels (Invitrogen). After fixation and drying, the gels were exposed to Kodak BioMax MR film with intensifying screens for 24 to 48 h. Densitometry on ^{35}S-labeled CG was performed using a BioRad GS-800 Calibrated Densitometer and QuantityOne software.

All experiments using EcoM-G cells were performed with cultures less than 3 months of age. EcoM-G clone EPSΔ1 ER 3.3 (Sykes and Kamps, 2001) cells were grown in RPMI1640, 10% FBS, 100U/ml each of penicillin and streptomycin, 10 ng/ml of recombinant murine GM-CSF and 1 μM β-estradiol. To induce EcoM-G differentiation, the cells were washed with PBS and seeded at 0.5 x 10^6 cells/ml in EcoM-G culture media without β-estradiol. The cathepsin C dipeptide nitrile inhibitors were added

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directly to the culture media 24 h later. Cells were harvested 48 h after seeding and lysed in lysis buffer B (20 mM Tris-HCl pH 7.5; 100 mM NaCl; 0.2% NP40). Debris was removed by centrifugation at 15,000 x g for 10 min, and supernatants were kept. NE, CG or Pr-3 enzymatic activities were measured as described in Méthot et al., 2007. Data was plotted using SigmaPlot 9.0 and sigmoidal curve fitting was performed using the Hill 4-parameter equation.

The TALL-104 gB activation cell-based assay was performed as following. TALL-104 cells (ATCC CRL-11386) were propagated in Iscove's Modified Dulbecco Medium (IMDM) supplemented with 20% FBS, 100U/ml each of penicillin, streptomycin and IL-2. The cells were seeded at 3 x 10^6 cells/ml in culture media in the presence of IL-12 (10 ng/ml) and cathepsin C inhibitors. The final DMSO concentration in each well was 0.5%. The cells were incubated at 37^{o}C 5% CO_2 in a humidified incubator, harvested by centrifugation 24 h later and washed twice in PBS before lysis with gB activity buffer (20 mM Tris-Cl pH 7.5; 50 mM NaCl; 1 mM EDTA; 5 mM DTT; 0.5% NP-40). Caspase-3 inhibitor (1 μM of M867; Méthot et al., 2004) and cathepsin C inhibitor (1 μM of compound 1; Méthot et al., 2007) were also added to the gB activity buffer to prevent post-lysis activation of gB and caspase-3-mediated cleavage of the synthetic substrate IEPD-AMC (Willoughby et al., 2002; Ewen et al., 2003). Cell debris was removed by centrifugation, and gB activity determined in the lysate by addition of IEPD-AMC to a final concentration of 200 μM. Release of AMC was measured spectrophotometrically using a Spectramax plate reader (Molecular Devices), with excitation at 355 nm and

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emission at 460 nm, for 10 min. The kinetic rates were calculated from the linear portion of the reaction.

Enzyme Occupancy Assays (EOA)- EcoM-G EOA were performed under conditions identical described for the serine protease cell-based assay, up to the lysis step. Before cell lysis, Ala-4-(^{125}I)Phe-DMK (2000 Ci/mmol) was added directly to the culture to a final concentration of 0.5 nM. The labeling reaction proceeded for 15 min and was stopped by the addition of a 2000-fold excess of Ala-4-(I)Phe-DMK. The cells were pelleted by centrifugation at 400 x g for 5 min washed with PBS containing 1 μM of Ala-4-(I)Phe-DMK and lysed with lysis buffer C (20 mM MES pH 6.0, 50 mM NaCl, 0.5% NP40) for 15 min on ice, before centrifugation at 15,000 x g. Cytosolic extracts were denatured by addition of reducing Laemmli buffer and boiling. Ala-4-(^{125}I)Phe-DMK-labeled proteins were resolved on 10-20% SDS-PAGE gels. The gels were fixed, dried and exposed to film for 6-24 h. TALL-104 EOA were also carried out under conditions identical to the serine protease cell-based assay, up to the lysis step. Labeling with Ala-4-(^{125}I)Phe-DMK and processing of the cell lysates was performed as described above for EcoM-G cells. U937 EOAs were performed under conditions similar to the pulse-chase assay. The cells were seeded in complete U937 culture media at 2 x 10^6 cells/ml, and incubated at 37^{o}C with 5% CO_2 in a humidified tissue culture incubator in the presence of cathepsin C inhibitors. After 4 h, Ala-4-(^{125}I)Phe-DMK (2000 Ci/mmol) was added to the culture to a final concentration of 0.5 nM for 15 min. The labeling reactions was stopped by the addition of a 2000-fold excess of Ala-4-(I)Phe-DMK. The cells were pelleted by centrifugation at 400 x g for 5 min washed with PBS containing 1 μM of Ala-4-(I)Phe-

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DMK and lysed 15 min on ice with lysis buffer B. Debris was removed by centrifugation at 15,000 x g for 10 min and cytosolic extracts were treated as described for the EcoM-G EOA. Densitometric quantitations were carried out with the QuantityOne software and a BioRad GS-800 densitometer. In all assays, care was taken to ensure the densitometric data was obtained with non-saturated film exposures and within the pixel saturation curves of the densitometer. In some cases multiple film exposures were obtained for comparison of densitometric data, and comparable results were obtained.

Western blotting- Protein extracts were prepared as described for the EOA, separated by SDS-PAGE and transferred to nitrocellulose membranes. Non-specific binding was reduced by incubating the membranes with blocking buffer [TBS supplemented with 0.1% Tween-20 (TBS-T) and 5% powdered milk] for 1 h at room temperature, before probing with antibodies. For cathepsin C, a 500-fold dilution of rabbit anti-mouse cathepsin C (gift from C. Pham) was prepared in blocking buffer. Anti-human granzyme B (Chemicon, Australia) and anti-β-actin (Sigma) were prepared at 1 μg/ml and 3000-fold, respectively, in blocking buffer. Antigen-antibody complexes were revealed with anti-rabbit-IgG-HRP (Amersham Biosciences) diluted 5000-fold in blocking buffer and developed with West-femto chemiluminescence reagents (Pearce) on Hyperfilm ECL (Amersham Biosciences).

Animals-Male Sprague-Dawley rats (250-300g; Charles River, St-Constant, Qc, Canada) and cathepsin C +/+ and -/- mice (Pham and Ley, 1999) were housed in a 12-hour light/dark cycle with free access to food and water. All procedures were carried out under

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appropriate Animal Care Committee approval in strict accordance to Merck and Co. animal care policies. Thioglycollate-induced mouse peritoneal macrophages were prepared as described in Boulet et al., 2004.

In vivo cathepsin C inhibition- The *ex vivo* cathepsin C EOA was performed using rats (n=3 per group) injected subcutaneously with compound 1 (10 mg/kg) or vehicle (100% PEG-200). The animals were euthanized by CO_2 inhalation 1 h after the injection. Femur bone marrow was flushed out with air, weighted and processed immediately for an *ex vivo* EOA. The marrow was suspended with an equal volume (w/v) of RPMI1640 supplemented with 10% FBS and 2 nM of Ala-4-(^{125}I)Phe-DMK. Labeling proceeded for 10 min and was stopped by the addition of 2 volumes of PBS containing 1 μM of Ala-4-(I)Phe-DMK. The cells were washed twice in PBS supplemented with 1 μM of Ala-4-(I)Phe-DMK and lysed in lysis buffer C as described in the cell-based EOA section. Protein concentration was measured using the BioRad assay and bovine-γ-globulin as standard and 75 μg were resolved by SDS-PAGE for autoradiography or transferred to nitrocellulose for western blotting. For the 2-week infusion experiment, rats were anesthetized with 2.5% isoflurane in oxygen and cannulated at the femoral vein by a small incision in the inguinal region. A silicone catheter (0.02”x 0.037’, Lomir) connected to a polyurethane catheter (PU-C30, 3 French, 80 cm, Instech Solomon) was inserted into the *vena cava*, exteriorized at the nape of the neck and clamped for the duration of the surgery. Baytril (enroflaxin) 10 mg/kg was administered on the day of the surgery and the following day. After one week of recovery with a sterile saline solution infusion, the rats were switched to solutions of either compound 1 (5 mg/kg/d in vehicle;

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n=6) or vehicle (10% PEG-200/0.9% NaCl; n=7) at a flow rate of 1 ml/kg/h. The animals were euthanized by CO_2 inhalation 2 weeks later, and blood and femur bone marrow were collected. Circulating leukocytes were prepared by lysing red blood cells with two consecutive hypotonic shocks in 0.2% NaCl. White blood cells were pelleted by centrifugation at 700 x g, washed once in PBS and frozen. Cell pellets were lysed in 20 mM Tris-Cl; 100 mM NaCl; 0.2% NP-40 for 15 min on ice, and debris removed by centrifugation at 15,000 x g for 10 min. Protein content was measured using the BioRad assay. Cytosolic extracts were subsequently used to measure NE, CG and Pr-3 activities with their respective substrates, as described above, with modified assay buffer (20 mM Tris-Cl pH 7.5; 1M NaCl; 0.2% NP40). To ascertain the specificity of the reaction, NE and CG assays were also carried out in the presence of their respective inhibitor (10 µM L-694,458 for NE; Davies et al., 1991 and 2 µM Calbiochem CG inhibitor I for CG). The reported NE data corresponds to the inhibitable portion of the MeOSuc-Ala-Ala-Pro-Val-AMC-cleaving activity, which typically amounted to 80% of the total. For CG, 100% of Suc-Ala-Ala-Pro-Phe-pNA cleavage was attributed to CG activity. Myeloperoxidase activity was determined in MPO assay buffer containing 50 mM K_2HPO_4 pH 6.0; 0.05% hexadecylmethylammonium bromide; 0.063 mg/ml o-dianisidine and 0.4 mM H_2O_2. Cytosolic extracts were added to the MPO assay buffer and absorbance at 450 nM was determined kinetically over 5 min. Rates were calculated on the linear portion of the curve.

Results

The cathepsin C active site probe detects active cathepsin C in intact murine cells

Ala-4-(^{125}I)Phe-DMK is a cathepsin C active site probe that binds covalently to multiple proteins in intact U937 cells, including the 23 kDa large subunit of cathepsin C (Méthot et al, 2007). To determine whether the other labeled peptides were related to cathepsin C, intact peritoneal macrophages, bone marrow suspension and whole blood from cathepsin C +/+ and -/- mice were exposed to Ala-4-(^{125}I)Phe-DMK, lysed and resolved by SDS-PAGE. Multiple proteins were labeled, with varying intensity and sizes, depending on the cell type. Only the 23 kDa protein, found in all cathepsin C$^{+/+}$ cell types examined, was consistently absent in cells originating from cathepsin C$^{-/-}$ mice (Fig. 1A). Two-dimensional gel electrophoresis, western blotting and competition with selective cathepsin C inhibitors showed that these other labeled peptides are not cathepsins B, L or S (data not shown).

EcoM-G is a murine pro-myelocytic cell line immortalized with an estrogen-regulated E2a/Pbx-1 fusion protein, and can differentiate into neutrophils when β-estradiol is withdrawn from the culture medium (Sykes and Kamps, 2001). NE, CG and Pr-3 are maximally induced 48 h following initiation of differentiation, and are dependent on cathepsin C for enzymatic activation (Méthot et al, 2007). This cell system is therefore well-suited to estimate fractional cathepsin C inhibition requirements to block serine protease activation. To test whether Ala-4-(^{125}I)Phe-DMK was able to differentiate between accessible and inhibited cathepsin C active sites, EcoM-G cells were seeded,

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differentiated and incubated for 24 h with the selective cathepsin C inhibitor compound 1 (Méthot et al., 2007; Fig. 2). Ala-4-(^{125}I)Phe-DMK (0.5 nM) was added to the culture media, and labeling was stopped after 15 min by exposing the cells to an excess of unlabeled Ala-4-Phe-DMK. The relative abundance of labeled proteins was not changed by β-estradiol (Fig. 1B lanes 1 and 3). In contrast, compound 1 prevented the labeling of the 23 kDa protein and a minor 27 kDa polypeptide of unknown identity (lanes 3 and 4). Western blotting showed that the cathepsin C p23 subunit is present in both undifferentiated and differentiated EcoM-G cells, and that its level of expression is not affected by compound 1 (Fig. 1C). Taken together, these data demonstrate that Ala-4-(^{125}I)Phe-DMK active site probe can be used to estimate the cathepsin C active site occupancy by specific, reversible inhibitors, in live cells.

Cathepsin C active site occupancy and inhibition of downstream serine proteases in cells

Next, we compared the occupancy of cathepsin C active sites present in EcoM-G with the percentage of NE inhibition, over a range of concentrations of cathepsin C inhibitors. Active site probes such as Ala-4-(^{125}I)Phe-DMK have been used to detect cysteine, serine and proteosome proteases in complex tissue extracts or living cells (Liu et al., 1999; Patricelli et al., 2001; Falgueyret et al., 2004; reviewed by Fonović and Bogyo, 2007). As with cell-surface receptor occupancy studies, a tracer amount of probe should be used to minimize the number of sites occupied by the probe itself and prevent competition with the reversible inhibitor. Labeling times should also be short to limit dissociation of the reversible inhibitor (Farde et al., 1992; Kapur et al., 2001). Labeling conditions that minimally perturb cathepsin C-reversible inhibitor equilibrium in EcoM-G cells were established by treating cells with Ala-4-(^{125}I)Phe with various concentrations, for different lengths of time. Increasing probe concentration or labeling time resulted in greater labeling intensity of all 4 polypeptides, including the cathepsin C p23 subunit (Fig. 1D). By densitometry, we estimate that less than 5% of the total cathepsin C active sites are labeled by 15 min of incubation with 0.5 nM of probe, assuming labeling achieved with 50 nM of probe is 100%. These conditions should therefore minimally affect binding equilibrium when a reversible inhibitor is present together with the probe. To evaluate how a range of cathepsin C active sites occupancy would impact serine protease processing, we compared fractional cathepsin C occupancy and serine protease activity in differentiating EcoM-G. The cells were seeded without β-estradiol to initiate differentiation, in duplicate. Cathepsin C inhibitors were added after 24 h, and incubated for another 24 h. In one set of assays, the cells were harvested and NE activity was measured in lysates. In the duplicate assay, Ala-4-(^{125}I)Phe-DMK (0.5 nM) was added to the cultures. Labeling was stopped with an excess of unlabelled Ala-4-(I)Phe-DMK 15 min later. The cells were lysed and cytosolic extracts were resolved by SDS-PAGE. Fig. 3 shows results obtained for a typical EcoM-G cathepsin C enzyme occupancy assay (EOA) with compounds 1 and 2 (Fig. 3A and B, respectively). Cathepsin C active sites were accessible and labeled by Ala-4-(^{125}I)Phe-DMK in the absence of inhibitor (DMSO control, lane 10). As the concentration of compound 1 increased, fewer cathepsin C active sites were available at equilibrium for capture by Ala-4-(^{125}I)Phe-DMK, and none were detected at the highest concentration of compound 1 tested. In this particular experiment (one of 3 independent EOA), half of the total available cathepsin C active sites were occupied by compound 1 or compound 2 at concentrations of 0.044 and 0.038 μM, respectively. We then compared the EOA IC_{50} with the inhibitor potencies for blockade of NE processing. The average IC_{50} values for NE activation for compounds 1 and 2 were 0.16 and 0.15 μM, respectively (Méthot et al., 2007), 3 to 5-fold more than the EOA IC_{50}. Superimposition of the EOA and NE activation dose-response curves (Fig. 3C and D) showed that inhibition by 50% of NE activation required 75-80% of cathepsin C active sites to be blocked. Several other cathepsin C inhibitors were tested. Those chosen (Fig. 2) are reversible and structurally similar, but span a range of potencies on purified recombinant cathepsin C (10-1000 nM IC_{50}). We reasoned that the relationship between occupancy and serine protease processing should be similar, regardless of the inhibitor potency. The cathepsin C EOA IC_{50} values were consistently lower than the NE processing IC_{50} values, on average, by 3.8-fold (Table 1). Thus, in differentiating EcoM-G cells, an excess of cathepsin C activity must be blocked in order to curb NE processing. For CG processing inhibition, fractional inhibition requirements must be greater still, since the CG processing IC_{50} values were always 3 to 5–fold greater than those for NE processing (Méthot et al., 2007).

The EOA and serine protease activation dose-response curves were compared in other cell types. U937 cells constitutively synthesize and process CG in a cathepsin C-dependent manner (Méthot et al., 2007). For all dipeptide nitriles tested, the EOA and CG activation IC_{50} values were very similar and overall there was no significant shift between the two assays (Table 2). Based on these results, we conclude that processing of

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the constitutively expressed CG in U937 cells does not require a high cathepsin C fractional inhibition.

Finally, we compared EOA and serine protease processing in the CD8+ T cell line TALL-104, which inducibly expresses granzyme B (gB) when treated with IL-12 (Cesano et al., 1993). Granzyme B was reported to require cathepsin C-mediated processing for activation (Pham and Ley, 1999; Thiele et al., 1997). Cleavage of the gB substrate IEPD-AMC was increased by 2.5-fold in lysates from TALL-104 cells treated with IL-12 for 24 h (Fig. 4A). This increase was enhanced if the cells were exposed to a caspase inhibitor (M867; Méthot et al., 2004). In contrast, a gB inhibitor (compound 20; Willoughby et al., 2002) reduced the IEPD-AMC cleavage. The irreversible cathepsin C inhibitor Gly-Phe-DMK (McGuire et al., 1993) also reduced gB activity, but did so indirectly since it did not inhibit purified gB enzyme (data not shown). Western blotting demonstrates that none of the inhibitors affected the total amount of gB protein (Fig. 4B). Furthermore, a slight decrease in gB protein mobility was often observed, possibly from the presence of two extra amino acids at the N-terminus that would be expected if cathepsin C activity was blocked. These data indicate that cathepsin C affects gB processing in these cells.

Active site occupancy and gB processing dose-responses were then compared for several cathepsin C inhibitors spanning a wide range of intrinsic potency. At high concentration, compound 1 fully occupied the cathepsin C active sites in TALL-104 (Fig. 4C). Surprisingly however, at least 10-fold more inhibitor was required to block 50% of the cathepsin C active sites compared to what was measured in EcoM-G and U937 cells

(Tables 1-3). Despite full occupancy of the active sites, gB processing was inhibited only to a maximum of 80% (Fig. 4D) and for all compounds tested, the maxima never exceeded 85%. The gB processing dose-response curves were shallow compared to the EOAs, and their IC_{50} values were always 3-5 fold greater than the EOA IC_{50} value (Table 3). These data and other recently published work (see discussion) suggest that although pro-gB can be activated by cathepsin C, at least one other protease is also able to activate the enzyme.

High cathepsin C fractional inhibition is required *in vivo* for blockade of neutrophil serine proteases

A comparison of IC_{50} values for EOA and inhibition of serine protease activity in cells pointed to various cathepsin C fractional inhibition requirements, depending on the cell type and the serine protease. Since granulocytes would be a major site of intervention for pharmaceutical cathepsin C inhibition, we measured NE and myeloperoxidase (MPO) activities in freshly isolated circulating leukocytes from cathepsin C +/+, +/- and -/- mice. No cathepsin C enzymatic activity was detected in leukocytes from cathepsin $C^{-/-}$, while about half of the wild type level was measured in cathepsin $C^{+/-}$ cells (Fig. 5A). In contrast, equal levels of NE activity were measured in leukocytes from cathepsin C +/- and +/+ mice. As expected (Adkison et al., 2002), cathepsin $C^{-/-}$ leukocytes contained less than 5% of wild type NE activity levels (Fig. 5B), and MPO activity was equal regardless of the genotype (Fig. 5C). Based in this, we expect that inhibition of serine protease activities via the pharmacological inhibition of cathepsin C *in vivo* will require greater than 50% active site occupancy.

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We then tested whether the requirement for high fractional inhibition precludes cathepsin C inhibition as a strategy to reduce the enzymatic activity of multiple serine proteases *in vivo*. NE, CG and Pr-3 are expressed and activated in the bone marrow during the promyelocyte stage (day 2-3 of neutrophil differentiation; Walker and Willemze, 1980; Fouret et al., 1989; Garwicz et al., 2005). Mature neutrophils are released from the bone marrow into the circulation after approximately 11-14 days of maturation, and, if left unstimulated, die after approximately 10 h (Walker and Willemze, 1980). We therefore reasoned that cathepsin C inhibition must take place in the bone marrow to be effective. To determine whether compound 1 can access cathepsin C in the bone marrow, an *ex vivo* whole cell EOA was performed. Rats (n=3/group) were dosed with compound 1 (10 mg/kg) or vehicle, and marrow from both femurs was recovered 1 h post-dosing. Drug levels were measured in marrow from one femur, while marrow from the other was rapidly suspended in a minimum volume of culture media containing Ala-4-(^{125}I)Phe-DMK and incubated for 10 min. Labeling of the 23 kDa cathepsin C subunit was evident in vehicle-treated animals, but significantly (>90%) reduced in compound 1-treated rats (Fig. 5D). A cathepsin C western blot shows similar levels of 23 kDa subunit in all samples (Fig. 5E). The concentration of compound 1 in the bone marrow was approximately 1.4 μM. We conclude that compound 1 blocks cathepsin C active sites *in vivo* in the bone marrow of rats. To test for the effect of compound 1 on serine protease activation *in vivo*, rats were cannulated in the femoral vein and infused with compound 1 (5 mg/kg/d) or vehicle. NE, CG, Pr-3 and MPO activities were measured in blood leukocytes after 2 weeks of infusion, at which time mature neutrophils that had been exposed to the cathepsin C inhibitor throughout their development should have entered the circulation. Compound 1 did not significantly affect the percentage of lymphocytes, monocytes and granulocytes in blood (not shown), and left MPO activity unchanged (Fig 5F). In contrast, NE, Pr-3 and CG activities were significantly reduced ($p<0.05$) by 80, 70 and 50%, respectively. The average bone marrow level of compound 1 after 2 weeks' dosing was 3.3 ± 0.5 μM. Thus, the simultaneous reduction of NE, CG and Pr-3 activities in granulocytes is achievable *in vivo* with a cathepsin C inhibitor in spite of a need for high enzyme occupancy.

Discussion

The inhibition of cathepsin C has the potential to reduce the activity of multiple pro-inflammatory serine proteases, and is therefore of interest for diseases with a high neutrophil burden, such as COPD and cystic fibrosis. We developed selective, reversible and non-toxic cathepsin C inhibitors, and showed that it is possible to inhibit simultaneously more than 90% of NE, CG and Pr-3 enzyme activities in the neutrophil cell line EcoM-G (Méthot et al., 2007). In this paper, we investigated further the serine protease inhibition requirements by developing cell-based enzyme occupancy assays (EOA) using the cathepsin C active site probe Ala-4-(^{125}I)Phe-DMK. We quantified the percentage of active site that must be blocked in order to affect serine processing in EcoM-G and U937 cells and extended our analysis to a novel cell-based assay that measures gB activation in the CD8+ TALL-104 cell line. In EcoM-G cells, which inducibly express NE, Pr-3 and CG, occupancy of 80% of the cathepsin C active sites reduced NE activity by half, whereas the same 50% inhibition of CG processing in U937

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cells was obtained when 50% of the cathepsin C active sites were occupied. In TALL-104 cells, IC_{50} values for gB inhibition were ~3-5 fold greater than that for cathepsin C active site occupancy. Thus, the cathepsin C fractional inhibition requirements for inhibition of downstream serine protease processing vary with cell line and target serine protease, but two examples point to a requirement for relatively high cathepsin C fractional inhibition. Although cathepsin C inhibitors significantly reduced gB activity in IL-12-treated TALL-104 cells, the dose-response relationships were shallow compared to those obtained with EcoM-G and U937 cells and the maxima of inhibition rarely surpassed 80%, despite complete cathepsin C active site occupancy. Granzyme B inhibition could not be maintained beyond 24 h as the enzyme activity gradually increased over time, without an increase in gB protein (J.R., unpublished observation). This contrasts with NE, CG and Pr-3 inhibition in EcoM-G cells, which was fully maintained for at least 3 d after the addition of compound 1 (Méthot et al., 2007). These data suggest that cathepsin C inhibition would not be an appropriate strategy to block granzyme B *in vivo*. Recent results from Mead et al. (2006) and Sutton et al., (2007) also indicate that gB activation is not fully dependent on cathepsin C.

The active site probe also permitted several other interesting observations. For example, compared to U937 and EcoM-G cells, 7-20-fold greater concentrations of compound 1 were necessary to block 50% of the cathepsin C active sites in TALL-104 cells. Compound 1 was equally stable under all 3 culture conditions, excluding compound degradation as an explanation. The number of cathepsin C active sites per cell (approximately 3000 for both U937 and TALL-104) and per assay (between 0.8 and 1.5

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fmoles for both assays) were similar and were a 100-fold lower than the number of active Ala-4-(^{125}I)Phe-DMK molecules present in the assay (J.R., C.B., unpublished data). We speculate that the difference of EOA IC_{50} values between TALL-104 and the neutrophil-like cell lines is a reflection of cellular permeability to compound 1 or the accumulation of compound 1 in lysosomes of a different nature. Compared to the intrinsic potency against purified cathepsin C, the EOA results were more predictive of the compound efficacy on serine protease processing. Potent cathepsin C inhibitors that suffered from low chemical stability were poor at blocking serine protease processing in cells, and accordingly, showed a high EOA IC_{50} value (J.R., unpublished data).

The biology of neutrophil maturation and serine protease activation implies that cathepsin C inhibition must occur in bone marrow to be therapeutically effective. Using an *ex vivo* EOA, we could show that cathepsin C active sites in the bone marrow of rats treated with compound 1 were almost completely blocked, with a compound 1 concentration of 1.4 μM in the femur marrow. In rats continuously infused for 2 weeks with compound 1, the bone marrow levels reached 3 μM (approximately 130-times the EcoM-G cell cathepsin C EOA IC_{50} value), and the net effect on the activities of NE, CG and Pr-3 was an 80%, 50% and 70% reduction, respectively. It is not known presently whether these percentages of inhibition are the maxima achievable, or if further inhibition is possible. Unfortunately, the chemical properties of compound 1 are not suitable to answer this question as more potent cathepsin C inhibitors would be needed. Compound 1 could not be dosed at higher levels due to its limited solubility in the intravenous vehicle. Nevertheless, to our knowledge, this is the first *in vivo* demonstration of the feasibility of

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inhibiting multiple serine proteases with a single cathepsin C inhibitor. The results also suggest that *in vivo* efficacy will require a very high fractional inhibition. Interestingly, similarly to what was observed in the EcoM-G cell-based assay (Méthot et al., 2007), *in vivo* leukocyte CG activity inhibition was also more difficult than NE and Pr-3 inhibition. Cathepsin C deficiency in humans causes Papillon-Lefèvre Syndrome (PLS; Toomes et al., 1999; Hart et al., 1999). Over 41 mutations in the human cathepsin C gene have been documented (Selvaraju et al., 2003) and most PLS patients tested exhibit an almost complete (>95%) loss of cathepsin C activity (Hewitt et al., 2003; Pham et al., 2004; deHaar et al., 2004; Nitta et al., 2005). In two published cases, relatives of PLS showed reduced cathepsin C activity but were asymptomatic (Nitta et al., 2005; Hewitt et al., 2003). One of these cases is particularly interesting, with a symptom-less sibling of a PLS patient having only 13% of the normal cathepsin C activity (Hewitt et al., 2003). The latter example supports the notion that very high cathepsin C fractional inhibition will be required for successful therapeutic intervention in humans.

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Figure Legends

Fig. 1 The active site probe Ala-4-(^{125}I)Phe-DMK binds to cathepsin C in intact murine cells. A- Autoradiogram of Ala-4-(^{125}I)Phe-DMK-labeled proteins from cathepsin C +/+ (lanes 1, 3, 5, WT) and -/- (lanes 2, 4, 6, KO) mice. Peritoneal macrophages (mϕ; lanes 1 and 2), femur bone marrow (BM; lanes 3, 4) and heparinated blood (lanes 5, 6) were exposed to 0.5 nM Ala-4-(^{125}I)Phe-DMK for 15 min before quenching with Ala-4-(I)Phe-DMK and resolution of lysates on SDS-PAGE. The 23 kDa protein was consistently absent in extracts from cathepsin C$^{-/-}$ mice. B- Autoradiogram of Ala-4-(^{125}I)Phe-DMK-labeled proteins from EcoM-G cells. EcoM-G cells were undifferentiated (+ β-estradiol, lanes 1, 2) or differentiated (- β-estradiol, lanes 3, 4) for 48 h, in the presence 10 μM of cmpd 1 for the final 24 h of differentiation. Ala-4-(^{125}I)Phe-DMK was added for the last 15 min and the cells were processed as described in Materials and Methods. Cmpd 1 prevented labeling of the 23 kDa protein (arrow). C- Western blot of cathepsin C in EcoM-G cells. EcoM-G cells were undifferentiated (+ β-estradiol, lanes 1, 2) or differentiated (- β-estradiol, lanes 3-7) for 48 h, in the presence of the indicated amount of cmpd 1. Neither differentiation, nor cmpd 1 changed the amount of the 23 kDa immunoreactive cathepsin C subunit (arrow). D- Autoradiogram of Ala-4-(^{125}I)Phe-DMK-labeled proteins from EcoM-G cells. The cells were differentiated for 48 h and exposed to the indicated time and concentration of Ala-4-(^{125}I)Phe-DMK. The final conditions for the EOA label less than 5% of the total cathepsin C active sites (compare lane 3, 4 with saturated labeling in lanes 7, 8).

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Fig. 2 Structures of novel dipeptide nitrile cathepsin C inhibitors used in this study. The range of potencies against purified cathepsin C varies from 10 nM (cmpd 2) to 1000 nM (cmpd 5) and is shown in Table 1.

Fig. 3 Cathepsin C EOA and NE processing inhibition in EcoM-G cells

A, B- autoradiography and densitometric data of Ala-4-(^{125}I)Phe-DMK-labeled EcoM-G cells treated with cmpd 1 (A) or cmpd 2 (B). Duplicate sets of EcoM-G cells were differentiated for 24 h, treated with the indicated concentration of cathepsin C inhibitor for an additional 24 h, and processed either for the NE activity assay or the cathepsin C EOA, as described in Materials and Methods. For both A and B, the autoradiogram is a representative example out of 3 independent experiments each for cmpd 1 and cmpd 2. The percentage of cathepsin C active sites occupied by was calculated from densitometric data, with 0% occupancy set with the DMSO control. The concentration of inhibitor which blocked 50% of the cathepsin C active sites (IC_{50}) is indicated in the lower right corner. C, D- Overlap of cathepsin C EOA and NE activity inhibition for cmpd 1 (C) and cmpd 2 (D). Data from 3 individual EOA each for cmpd 1 and 2 were combined, whereas for NE activity, data from 7 independent experiment (cmpd 1) or 5 experiments (cmpd 2) were combined. Error bars represent the SEM. A 50% inhibition of NE activity requires blockade of 80% of the cathepsin C active sites.

Fig. 4 Cathepsin C EOA and Granzyme B processing inhibition in TALL-104 cells

Granzyme B (gB) enzymatic activity (IEPD-AMC cleavage) in lysates from TALL-104 cells treated for 24 h with IL-12 and the indicated compound. B- Granzyme B western

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blot of TALL-104 lysates used to generate enzyme activity data in A. The gB inhibitor cmpd and the cathepsin C inhibitor GF-DMK reduced gB activity without affecting gB protein levels. Note the slight decrease gB mobility in lysates from GF-DMK-treated cells. C, D- Autoradiography and densitometric data of Ala-4-(^{125}I)Phe-DMK-labeled TALL-104 cells treated with cmpd 1. Duplicate sets of TALL-104 cells were treated with IL-12 and cmpd 1 for 24 h and processed either for the gB activity assay or the cathepsin C EOA, as described in Materials and Methods. The autoradiogram is a representative example from 2 independent experiments. The percentage of cathepsin C active sites occupied by was calculated from densitometric data, with 0% occupancy set with the DMSO controls. The concentration of cmpd 1 which blocked 50% of the cathepsin C active sites (IC_{50}) is indicated in the lower right corner. D- Overlap of cathepsin C EOA and gB activity inhibition for cmpd 1, with combined data from 2 independent EOA and 7 independent gB activity assays. Error bars represent SEM.

Fig. 5 High fractional inhibition of cathepsin C required for inhibition of serine protease processing *in vivo*. Cathepsin C (A), NE (B) and MPO (C) enzymatic activities in circulating leukocyte lysates from cathepsin C +/+, +/- and -/- mice. The data shown is the average from 4 individual animals ± SEM. D- Autoradiograph of Ala-4-(^{125}I)Phe-DMK-labeled bone marrow obtained from cmpd 1 (n=3) or vehicle (n=3) injected rats. The samples were processed as described in the Materials and Methods section. The labeled cathepsin C p23 subunit is indicated by an arrowhead. E- Cathepsin C western blot of extracts shown in (D). Ala-4-(^{125}I)Phe-DMK failed to label active cathepsin C in the presence of cmpd 1 (D) despite the presence of cathepsin C protein, indicating *bona*

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fide active site occupancy by compound 1. F- Neutral serine proteases (NE, CG and Pr-3) and MPO enzymatic activities in lysates of circulating leukocytes from rats treated for 2 weeks with compound 1 (n=6) or vehicle (n=7). The reported NE data corresponds to the L-694,458-inhibitable portion of the MeOSuc-Ala-Ala-Pro-Val-AMC-cleaving activity, which typically amounted to 80% of the total. For CG, 100% of Suc-Ala-Ala-Pro-Phe-pNA cleavage was attributed to CG activity (See Materials and Methods for details). Statistically significant reductions of NE, CG and Pr-3 but not MPO activities were measured in the compound 1-treated rats. Error bars represent SEM.

Table 1. Cathepsin C inhibitor potencies (IC_{50}) on recombinant enzyme, cell-based neutrophil elastase activation and active site occupancy in EcoM-G cells

Compound	**Enzyme Potency (nM)**	**Cell-based Potency (µM)**		**Ratio**
	Cathepsin C	**Cathepsin C EOA**	**NE Activation**	**NE act/EOA**
Compound 1	**14 ± 1***	**0.023 ± 0.011**	**0.16 ± 0.03***	**7.0**
Compound 2	**10 ± 2**	**0.026 ± 0.006**	**0.15 ± 0.03**	**5.8**
Compound 3	**347 ± 72**	**2.23 ± 0.80**	**5.49 ± 1.17**	**2.5**
Compound 4	**793 ± 160**	**3.05**	**8.20 ± 0.75**	**2.7**
Compound 5	**986 ± 82**	**3.70**	**12.62 ± 2.68**	**3.4**
Compound 6	**11 ± 3**	**0.05**	**0.12 ± 0.02**	**2.2**
Compound 7	**722 ± 130**	**7.20**	**18.0 (n=2)**	**2.5**

EcoM-G cells were differentiated for 24 h before addition of inhibitors to the cell culture media. The cells were harvested 24 h later and processed either for NE activity or cathepsin C enzyme occupancy assay (EOA), as described in Materials and Methods. *Values previously reported in Méthot et al., 2007. SEM are indicated for all compounds tested in at least 3 independent assays. The average IC_{50} for each

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compound was calculated by averaging single IC_{50} values obtained in individual experiments.

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Table 2. Cathepsin C EOA and cathepsin G processing IC_{50} in U937 cells

Compound	**Cell-based Potency (µM)**		**ratio**
	Cathepsin C EOA	**CG Activation**	**CG Act./EOA**
Compound 1	**0.09 (n=2)**	**0.15 (n=2)***	**1.69**
Compound 2	**0.12 (n=3)**	**0.09 (n=2)**	**0.73**
Compound 3	**2.10**	**1.2**	**0.57**
Compound 4	**9.00**	**9.00**	**1.00**
Compound 5	**11.40**	**10.50**	**0.92**
Compound 7	**2.18 (n=2)**	**3.00**	**1.37**

*Value previously reported in Méthot et al., 2007. The number of replicates for each compound tested more than once in the assays is indicated in parenthesis.

Table 3. Cathepsin C EOA and granzyme B inhibition IC_{50} in TALL-104 cells

Compound	**Cell-based Potency (µM)**		**ratio**
	Cathepsin C EOA	**gB Activation**	**gB Act./EOA**
Compound 1	**0.89 ± 0.15**	**2.45 ± 0.37**	**2.9**
Compound 2	**0.75 (n=2)**	**2.75 ± 0.26**	**3.7**
Compound 3	**11.0**	**13.8 (n=2)**	**1.3**
Compound 4	**>50**	**38 (n=2)**	**n.a.**
Compound 5	**>50**	**>50**	**n.a.**
Compound 6	**0.55**	**2.42**	**4.4**
Compound 7	**29.0**	**41 (n=2)**	**1.4**

Data ± SEM values are indicated for all compounds tested in at least 3 independent assays. The average IC_{50} for each compound was calculated by averaging single IC_{50} values obtained in individual experiments.

Fig. 1

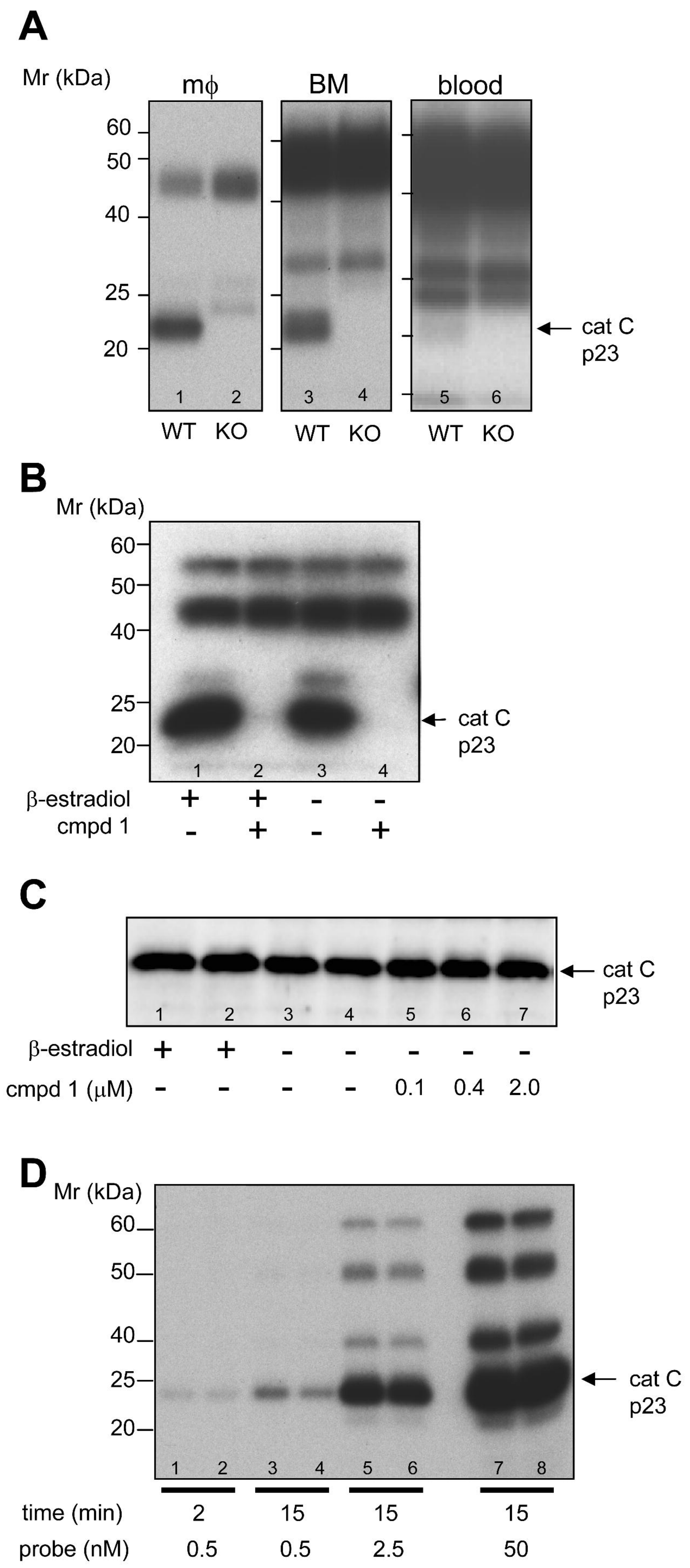
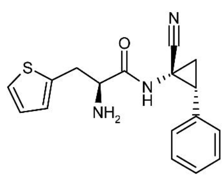
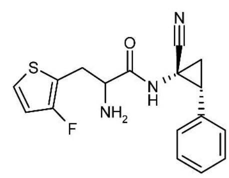

A
Mr (kDa)
mϕ
BM
blood
60
50
40
25
20
1
2
3
4
5
6
WT
KO
WT
KO
WT
KO
cat C
p23
B
Mr (kDa)
60
50
40
25
20
1
2
3
4
cat C
p23
β-estradiol + + - -
cmpd 1 - + - +
C
1
2
3
4
5
6
7
cat C
p23
β-estradiol + + - - - - -
cmpd 1 (μM) - - - - 0.1 0.4 2.0
D
Mr (kDa)
60
50
40
25
20
1
2
3
4
5
6
7
8
cat C
p23
time (min) 2 15 15 15
probe (nM) 0.5 0.5 2.5 50

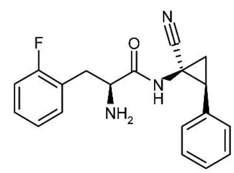
Fig. 2



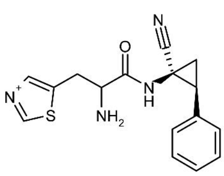
compound 1



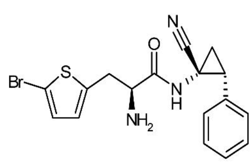
compound 2



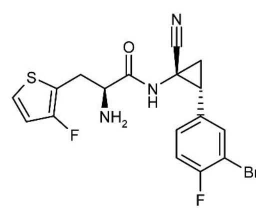
compound 3



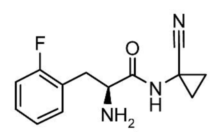
compound 4



compound 5



compound 6



compound 7

Fig. 3

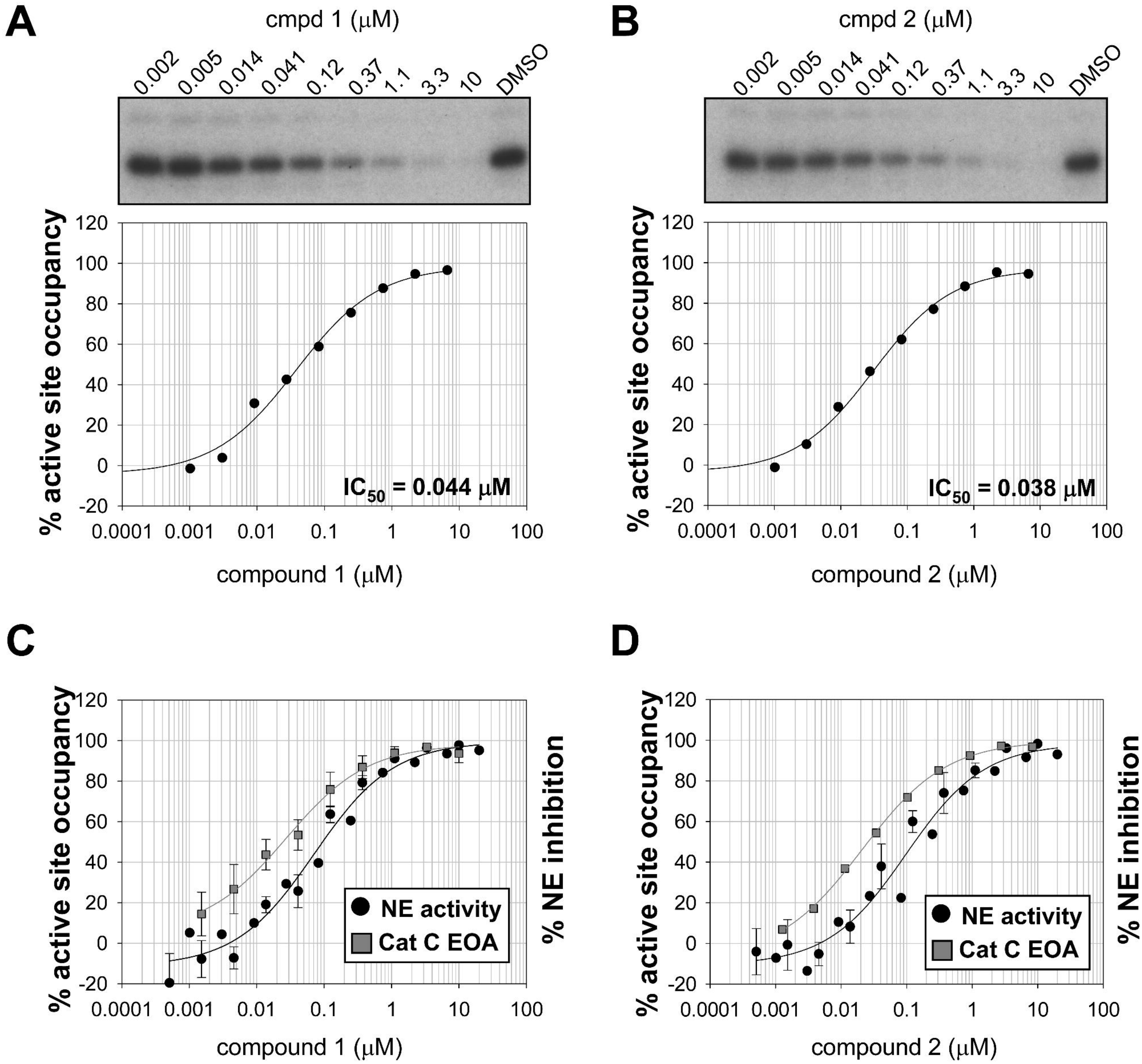

A
cmpd 1 (μM)
0.002 0.005 0.014 0.041 0.12 0.37 1.1 3.3 10 DMSO
% active site occupancy
IC50 = 0.044 μM
compound 1 (μM)
B
cmpd 2 (μM)
0.002 0.005 0.014 0.041 0.12 0.37 1.1 3.3 10 DMSO
% active site occupancy
IC50 = 0.038 μM
compound 2 (μM)
C
% active site occupancy
% NE inhibition
NE activity
Cat C EOA
compound 1 (μM)
D
% active site occupancy
% NE inhibition
NE activity
Cat C EOA
compound 2 (μM)

Fig. 4

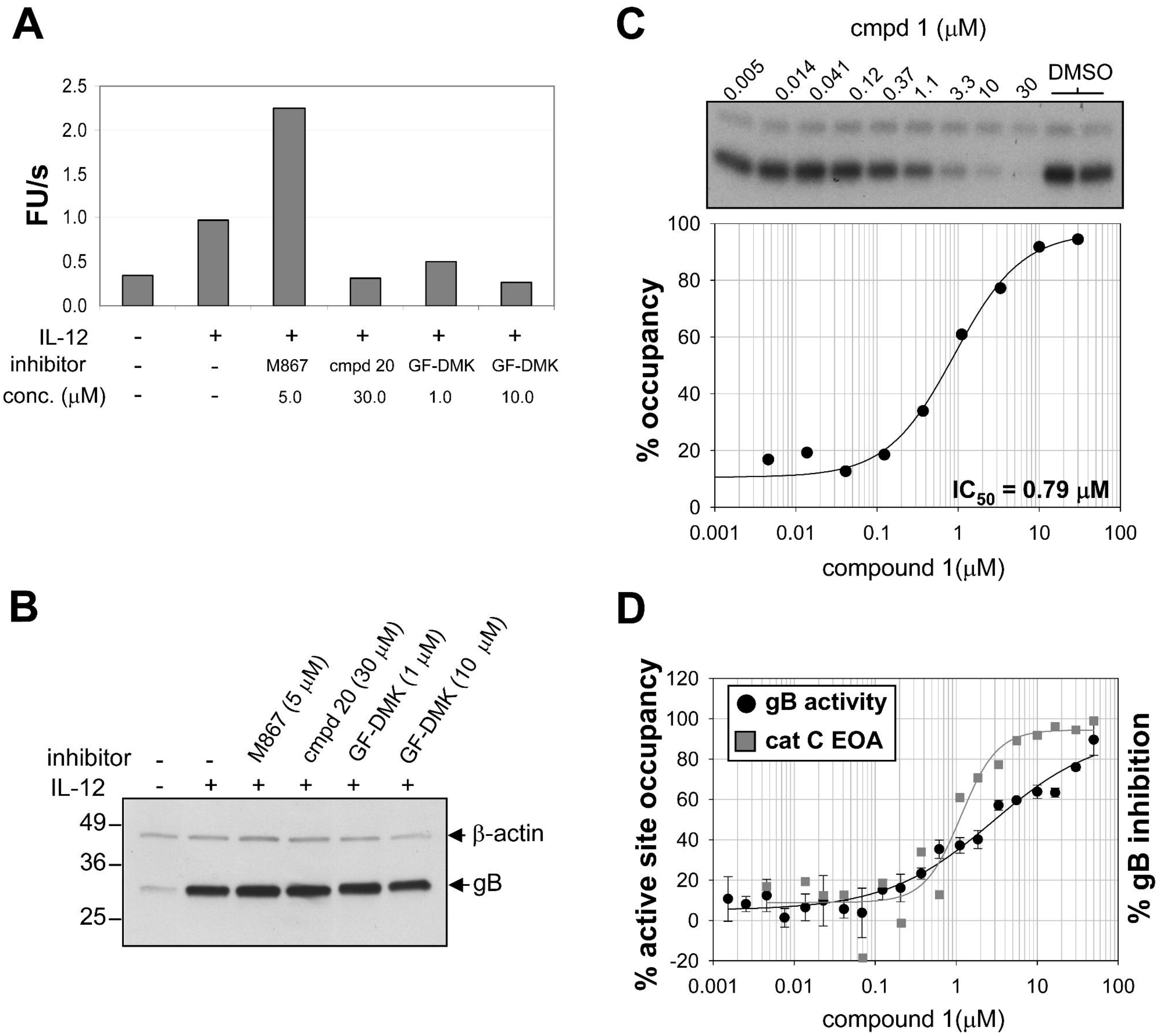
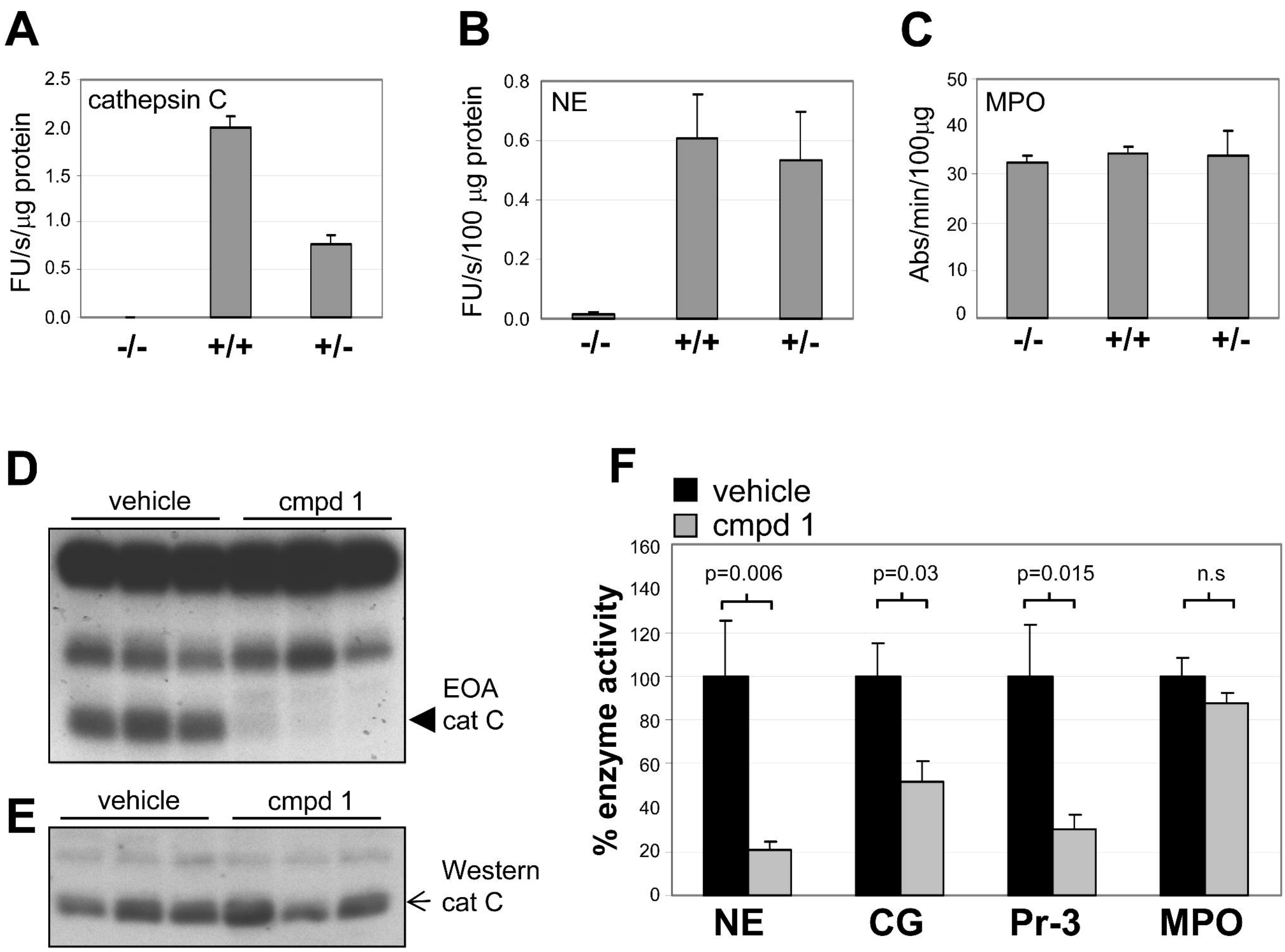

A
FU/s
2.5
2.0
1.5
1.0
0.5
0.0
IL-12 - + + + + +
inhibitor - - M867 cmpd 20 GF-DMK GF-DMK
conc. (μM) - - 5.0 30.0 1.0 10.0
B
inhibitor - - M867 (5 μM) cmpd 20 (30 μM) GF-DMK (1 μM) GF-DMK (10 μM)
IL-12 - + + + + +
49
36
25
β-actin
gB
C
cmpd 1 (μM)
0.005 0.014 0.041 0.12 0.37 1.1 3.3 10 30 DMSO
% occupancy
IC50 = 0.79 μM
compound 1(μM)
D
gB activity
cat C EOA
% active site occupancy
% gB inhibition
compound 1(μM)

Fig. 5


A
cathepsin C
FU/s/μg protein
2.5
2.0
1.5
1.0
0.5
0.0
-/-
+/+
+/-
B
NE
FU/s/100 μg protein
0.8
0.6
0.4
0.2
0.0
-/-
+/+
+/-
C
MPO
Abs/min/100μg
50
40
30
20
10
0
-/-
+/+
+/-
D
vehicle
cmpd 1
EOA
cat C
E
vehicle
cmpd 1
Western
cat C
F
vehicle
cmpd 1
% enzyme activity
160
140
120
100
80
60
40
20
0
p=0.006
p=0.03
p=0.015
n.s
NE
CG
Pr-3
MPO